Dendritic Cell-Based Therapeutic Vaccination against Myeloma: Vaccine Formulation Determines Efficacy against Light Chain Myeloma

Sharon Cohen, Joseph Haimovich and Nurit Hollander

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Multiple myeloma is an incurable neoplastic disease characterized by the outgrowth of monoclonal plasma cells. Although high-dose chemotherapy followed by stem cell transplantation may result in complete remission, relapse remains a major problem. Therefore, novel approaches to control residual disease are needed. One such approach may be based on Id vaccination. Ig Id of malignant B cells are tumor-specific Ags uniquely expressed in a given B cell or plasma cell malignancy. Hence, the Id has been exploited as a target for active immunotherapy. The first generation of Id vaccines consisted of purified Id or Id protein conjugated to an immunogenic carrier protein, such as keyhole limpet hemocyanin (KLH),2 injected together with an immunologic adjuvant. Vaccination with these vaccines conferred protection against tumor challenge in a number of lymphoma and myeloma animal models (1–4). Based on these preclinical results, immunization with autologous Id has been initiated in clinical trials to control residual disease in B cell lymphoma and multiple myeloma (5–7). Phase II clinical trials with an Id-KLH plus GM-CSF vaccine in patients with follicular lymphoma resulted in encouraging immunological and clinical outcomes (8–10). As a result, Phase III clinical trials are currently evaluating the clinical benefit of the Id-KLH vaccine (8–10).

In a more recent approach, based on the central role of dendritic cells (DCs) in the initiation of immune responses (11), DCs have been used to augment the potency of Id vaccines. As the most powerful APCs, DCs take up, process, and present Ag in the context of costimulatory signals required for activation of CD4+ and CD8+ T cells. Studies in patients with non-Hodgkin’s lymphoma treated with DCs loaded with tumor-derived Id protein showed high-frequency antitumor immune responses and clear clinical responses (12, 13). Randomized controlled clinical trials are required to definitively answer the question of clinical benefit induced by DC-based Id vaccination in non-Hodgkin’s lymphoma.

Id vaccination in multiple myeloma has been less successful compared with lymphoma. Targeting the Id in myeloma depends on efficient cell-mediated immune responses because, unlike anti-Id Abs, T cells would not be blocked by the large amounts of circulating paraprotein and would not depend on expression of the native protein on the tumor cell surface. Hence, optimal strategies for Id vaccination in myeloma require induction of effector T cells, which may be best achieved by the use of DCs. Although Id-specific immune responses were detected with variable frequency in myeloma patients immunized with Id-pulsed DCs, clinical responses were poor even when DC administration was followed by boosts of Id-KLH in adjuvant (14–18). Hence, new DC-based immunization strategies are needed. One such strategy may use Id-KLH-pulsed DCs instead of the commonly used Id-pulsed DC formulation. It has been demonstrated that linkage of the Id to a foreign carrier protein enhances the immunogenicity of a pulsed DC vaccine (19). Another strategy may overcome the weak immunogenicity of idiotypic epitopes by using DCs loaded with whole tumor cells to elicit immunity against various Ags expressed by the tumor. Animal studies (20–22) and early clinical trials (23–25) have shown that DCs loaded with apoptotic tumor cells or freeze-thaw lysates can elicit antitumor immunity.
We have previously demonstrated that Id-KLH-pulsed DCs, unlike Id-pulsed DCs, induced Id-reactive CD8+ T cells and protection against myeloma tumor challenge in mice (26). Based on these results, we further investigated vaccination in a therapeutic model, in which mice carrying advanced plasma cell tumors were treated with chemotherapy, followed by a DC-based vaccine. Comparative studies with different vaccine formulations showed that s.c. injection of Id-KLH-pulsed DCs, s.c. injection of DCs loaded with irradiated tumor cells, and intratumoral injection of naive DCs were similarly effective in mediating tumor regression and long-term survival. However, whereas the Id-KLH-DC vaccine was inefficient against myeloma cells that lost expression of the Ig H chain, intratumoral injection of naive DCs and s.c. injection of DCs loaded with tumor cells were highly effective against cells producing L chains only. This may be of particular importance for patients with L chain myeloma and nonsecretory myeloma.

Materials and Methods

Mice and cell lines

Eight-week-old female BALB/c mice were obtained from the animal facility of Tel Aviv University. All procedures were approved by the Institutional Animal Care and Use Committee. JLμs and JLδμ are transfectoma cell lines derived from the H-chain-deficient A1 lung-producing J558L plasmacytoma (27) transfected with the 17.2.25 μ H chain (28). JLμs and JLδμ were obtained by transfection with plasmids coding for either the secreted (μs) or the membrane (δm) form of the 17.2.25 μ H chain (29). Thus, whereas JLμs cells secrete IgM, JLδμ cells produce a substantial amount of IgG, which is neither deposited on the cell surface nor secreted (30). R2.438.8 is a hybridoma cell line secreting a rat mAb against the 17.2.25 VHDJH Id (31). It was provided by T. Imanishi-Kari (Tufts University, Boston, MA). Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME.

Isolation and coupling of Id protein

JLμs cells were grown as ascites. Because the specificity of the JLμs IgM is directed to the 4-hydroxy-3-nitrophenyl hapten, it was purified from ascites by affinity chromatography on a nitrophenyl-BSA column, as previously described (32). The JLμs IgM was coupled to KLH using 0.1% glutaraldehyde, as described (33).

Generation and loading of DCs

DCs were obtained from mouse bone marrow, as described by Lutz et al. (34). Briefly, bone marrow was flushed from the femurs and tibia of mice, and resuspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, and 20 ng/ml murine rGM-CSF (ProSpec-Tany TechnoGene). Cells were plated in bacteriological petri dishes (Falcon No. 1029; BD Biosciences) and then fed with the same medium on days 3 and 6. On day 8, 2-ME, and 20 ng/ml murine rGM-CSF (ProSpec-Tany TechnoGene), and then fed with the same medium on days 3 and 6. On day 8, nonadherent cells were collected, centrifuged at room temperature, resuspended in medium supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME.

In vivo tumor treatment

Mice were injected s.c. in the right flank with 5 × 106 JLμs tumor cells. When tumors reached ~1.5 cm in diameter, mice were treated with cyclophosphamide by i.p. injection of 50 mg/kg body weight. On days 3 and 8 postchemotherapy, 8 × 105 Id-KLH-loaded DCs were injected s.c. in the left flank. The systemic chemotherapy resulted in tumor rejection in only 40–50% of treated animals. In 50–60% of tumor-bearing animals, chemotherapy resulted in transient tumor regression, followed by relapse and death (Fig. 1A). However, vaccination with Id-KLH-loaded DCs after chemotherapy led to complete regression and long-term survival of 100% of animals (p < 0.005 vs chemotherapy alone). Injection of Ag-loaded DCs without prior chemotherapy had no antitumor effect, indicating that cytoreduction of advanced-stage tumors is a prerequisite for effective vaccination. As a control for Id specificity of the antitumor response, mice bearing the J558L plasmacytoma were similarly treated. As shown in Fig. 1B, cytoreduction plus vaccination with Id-KLH-loaded DC had no advantage in comparison with chemotherapy alone.

Flow cytometry

Cells were incubated for 1 h at 4°C with 2% paraformaldehyde, spun, and incubated for 15 min at 37°C with 0.2% Tween 20. Cells were then washed with PBS and incubated for 45 min at 4°C with the anti-Id mAb R2.438.8. Following washes with 0.1% Tween 20, cells were incubated with FITC-conjugated mouse anti-rat IgG (Jackson Immunoresearch Laboratories). Cells were washed with PBS supplemented with 5% FCS and analyzed on a FACSort flow cytometer (BD Biosciences).

Western blot analysis

Cells were lysed with 1% Triton X-100 in 0.15 M NaCl and 25 mM Tris-HCl (pH 8.0) containing protease inhibitors. Lysates were centrifuged at 12,000 × g for 15 min to remove insoluble material. Lysates were resolved under reducing conditions by 10% SDS-PAGE and transferred to nitrocellulose membranes. Blocked membranes were incubated with the primary Abs rabbit anti-mouse A1 chain (MB Biomedicals), goat anti-mouse IgM (36), or rabbit anti-calnexin (StressGen Biotechnologies), followed by incubation with HRP-conjugated protein A (Sigma-Aldrich) or HRP-conjugated mouse anti-goat IgG (Jackson Immunoresearch Laboratories). The probed proteins were visualized by ECL.

Statistical analysis

Differences between survival curves were assessed using the Kaplan-Meier method with the log-rank test. Values at p < 0.05 were considered statistically significant.

Results

Vaccination with Id-KLH-loaded DCs induces regression of established tumors in combination with chemotherapy

Mice were injected s.c. in the right flank with 5 × 106 JLμs tumor cells. When tumors reached ~1.5 cm in diameter, mice were treated with cyclophosphamide by i.p. injection of 50 mg/kg body weight. On days 3 and 8 postchemotherapy, 8 × 105 Id-KLH-loaded DCs were injected s.c. in the left flank. The systemic chemotherapy resulted in tumor rejection in only 40–50% of treated animals. In 50–60% of tumor-bearing animals, chemotherapy resulted in transient tumor regression, followed by relapse and death (Fig. 1A). However, vaccination with Id-KLH-loaded DCs after chemotherapy led to complete regression and long-term survival of 100% of animals (p < 0.005 vs chemotherapy alone). Injection of Ag-loaded DCs without prior chemotherapy had no antitumor effect, indicating that cytoreduction of advanced-stage tumors is a prerequisite for effective vaccination. As a control for Id specificity of the antitumor response, mice bearing the J558L plasmacytoma were similarly treated. As shown in Fig. 1B, cytoreduction plus vaccination with Id-KLH-loaded DC had no advantage in comparison with chemotherapy alone.

Long-term surviving mice (5 mo postchemotherapy and post-vaccination) were rechallenged with the same dose of JLμs tumor cells. As shown in Fig. 1C, surviving mice were able to resist a rechallenge (p < 0.001), indicating that combined treatment with chemotherapy and Id-KLH-loaded DCs induced immune-mediated tumor rejection with long-term memory.

Intratumoral injection of unloaded DCs induces regression of tumors in combination with chemotherapy

In addition to vaccination with Id-KLH-loaded DCs, we investigated a different DC-based immunotherapy approach that does not require ex vivo Ag loading of DCs. Mice were injected s.c. with 5 × 106 JLμs tumor cells. When tumors reached ~1.5 cm in diameter, mice were treated with cyclophosphamide by i.p. injection of 50 mg/kg body weight. On days 3 and 8 postchemotherapy,
2 × 10⁶ naive DCs were injected directly into the tumor. Similarly to vaccination with Id-KLH-loaded DCs, intratumoral injection of naive DCs after chemotherapy was superior to chemotherapy alone (p < 0.01), leading to complete regression and long-term survival of 100% of animals (Fig. 2). Intratumoral injection of DCs without prior chemotherapy had no antitumor effect, indicating that the mere presence of DCs at the tumor site is not sufficient to induce an antitumor immune response.

To determine whether combination of chemotherapy and intratumoral DC injection induced an antitumor T cell response, we performed IFN-γ-ELISPOT assays. Four months postchemotherapy and postvaccination, spleen cells of surviving mice were incubated with tumor cells. As shown in Fig. 3, T lymphocytes of surviving mice reacted specifically to JLμs tumor by IFN-γ secretion (p < 0.00001).

**Elimination of Id-negative variant tumor cells induced by DC treatment**

We occasionally observe emergence of Id-negative cells in JLμs and other lines of plasma cell tumors. We therefore investigated the therapeutic effect of DC-based vaccines on tumors consisting of mixed populations of Id-positive and Id-negative cells. Mice were injected s.c. in the right flank with 5 × 10⁶ JLμs tumor cells consisting of mixed wild-type and Id-variant cells (Fig. 4A). The proportion of Id-positive and Id-negative cells in the injected mixture was 28 and 72%, respectively. When tumors reached ~1.5 cm in diameter, mice were treated with cyclophosphamide. On days 3 and 8 postchemotherapy, 2 × 10⁶ naive DCs were injected directly into the tumor of CY-treated mice or CY-untreated mice (DC alone). Each experimental group consisted of 10 mice.
The distinct outcome of the two vaccination strategies could result either from the distinct routes of DCs application (intratumoral vs distant s.c. site) or from the distinct nature of loaded tumor Ags (in situ loading of apoptotic or necrotic tumor cells vs ex vivo loading of Id-KLH protein). To answer this question, DCs loaded ex vivo with irradiated Id\textsuperscript{+}/Id\textsuperscript{-} mixed tumor cells were injected s.c. in the left flank of cyclophosphamide-treated tumor-bearing mice. The results indicated that distal s.c. injection of DCs loaded with irradiated tumor cells was as effective as intratumoral injection of naive DCs in the rejection of tumors containing Id-negative variant cells (Fig. 4B). The s.c. injection of unloaded DCs was ineffective. Thus, DCs loaded with dying tumor cells either in situ or ex vivo are highly effective in the induction of an antitumor response that leads to elimination of Id-negative variant cells.

The tumor identity in mice injected with mixed populations of Id-positive and Id-negative cells was analyzed for Id expression at the experiment end point. As shown in Fig. 5, tumors consisting of mixed populations at the time of inoculation converted to Id-negative populations in Id-KLH-DC-vaccinated mice. Hence, selective depletion of Id-positive tumor cells had occurred.

Resistance to mixed populations of wild-type and Id-variant tumor cells could result from a bystander effect, whereby an Id-specific response against the wild-type cells indirectly affected the Id-negative cells. Alternatively, resistance could reflect an Id-independent response to tumor Ags other than Id. To determine whether the antitumor response, induced by tumor cell-loaded DCs, was dependent on Id expression, we used tumors consisting exclusively of Id-negative cells, derived by cloning of mixed tumor cell populations. Mice were injected s.c. in the right flank with 5 × 10\textsuperscript{6} Id-negative JL\textsubscript{mu} tumor cells (Fig. 6A). When tumors reached ~1.5 cm in diameter, mice were treated with cyclophosphamide. On days 3 and 8 postchemotherapy, mice were treated by Id-KLH-loaded DCs injected s.c. in the left flank, by Id-negative tumor cell-loaded DCs injected s.c. in the left flank, or by naive DCs injected directly into the tumor. As shown in Fig. 6B, intratumoral injection of naive DCs and s.c. injection of Id-negative tumor cell-loaded DCs induced complete regression and long-term control.
survival of 100% of treated animals \( (p < 0.001 \text{ for both treatments}) \). In contrast, vaccination with Id-KLH-loaded DCs was ineffective, resulting in survival rates similar to those obtained in mice treated with chemotherapy alone (data for chemotherapy alone not shown). Thus, antmyeloma immunity induced by vaccination with tumor cell-loaded DCs did not depend on expression of Id.

It should be noted that the Id-negative JLm cells were more aggressive than the Id-positive cells. Thus, chemotherapy resulted in only transient tumor regression, followed by relapse and death of all mice. Nevertheless, combined treatment with chemotherapy and DCs mediated 100% tumor regression and long-term survival (Figs. 4 and 6).

It should also be noted that although whole tumor cell vaccines may have the potential to induce autoimmunity, we did not observe any signs of autoimmunity or illness (behavioral changes, weight loss, hair loss, and wasting). All survivor animals appeared healthy up to 6 mo after treatment.

Id-variant JLm cells express Ig L chains only

Although most multiple myeloma cells are characterized by a monoclonal Ig, in up to 20% of myeloma cases only L chain is detected due to absence of H chain synthesis. To study whether the Id-negative JLm tumor cells are phenotypically similar to L chain-only multiple myeloma cells, lysates of JLm wild-type and variant cells were analyzed by Western blotting for expression of A L chain and \( \mu \) H chain. This analysis revealed that the Id-negative cells lost expression of the Ig H chain, but maintained production of the L chain (Fig. 7).

The anti-Id response is directed against the Ig H chain

Because vaccination with Id-KLH-loaded DCs provided no protection against the H chain-deficient JLm variant cells, it was suggested that the antitumor response mediated by this vaccine depends on expression of H chains. To determine the specificity of the antitumor response, mice were immunized twice by s.c. injection of Id-KLH-pulsed DCs. Immunized mice were challenged with J558L, JLm, or JLm cells. The J558L cell line is a H chain-deficient \( \lambda \) L chain-producing plasmacytoma (27). JLm and JLm cell lines were derived from J558L by transfection with the 17.2.25 \( \mu \) H chain (28), using plasmids coding for either the secreted (\( \mu \)s) or the membrane (\( \mu \)m) form of the 17.2.25 \( \mu \) H chain (29). As shown in Fig. 8, vaccination did not elicit resistance to the J558L tumor. In contrast, vaccinated mice were highly resistant to both JLm and JLm tumors \( (p < 0.001) \). Thus, tumor protection following immunization with the myeloma Ig depended on H chain expression by the tumor.

Discussion

The main objective of this study was to compare the therapeutic efficacy of different DC-based vaccines. Intratumoral injection of DCs was compared with a whole tumor cell-DC vaccine and to the more traditional Id-KLH-DC vaccine. For the cell-based vaccine, DCs were pulsed with irradiated tumor cells rather than with tumor cell lysates, because irradiated apoptotic myeloma cells were found to be a superior source of Ag compared with tumor lysates for ex vivo DC-mediated induction of myeloma-specific T cells (37). We have previously demonstrated that vaccination with Id-KLH-DC before tumor challenge elicited a significant anti-JLm tumor protection that was mediated entirely by CD8\(^+\) T cells (26).

In this work, we studied the antitumor effect of DC vaccines in mice bearing large pre-existing tumors. In this setting, when applied as a single therapeutic agent, the three different DC vaccines had no antitumor effect. However, application of the DC vaccines in combination with chemotherapy resulted in complete long-term tumor regression, indicating that cytoreduction of advanced-stage tumors is a prerequisite for effective vaccination. Cytoreduction...
may be required, among other reasons, to lower the level of suppressive factors secreted by tumor cells. For instance, the cytokine TGF-β that is produced by numerous tumor types, including multiple myeloma, has been demonstrated to suppress antitumor immune responses by inhibiting DC maturation and migration, and by impairing T cell effector functions (38, 39). Thus, it has been reported that intratumoral injection of DCs, which was not preceded by chemotherapy, required concomitant application of anti-TGF-β neutralizing Abs (38). In addition to cytodestruction, chemotherapy may enhance immune reactivity. Many antineoplastic drugs, including cyclophosphamide, can exert both immunosuppressive and immunomodulating effects, depending on the dosage and the temporal relationship between drug administration and Ag challenge (40). It has been shown that a single administration of cyclophosphamide depleted CD4+CD25+ regulatory T cells and delayed tumor growth (41). Moreover, cyclophosphamide has been shown to augment the antitumor effect of DC vaccines by reducing the level of regulatory T cells and by inducing IFN-γ production (42).

Unlike the whole tumor cell-DC vaccine and the Id-KLH-DC vaccine, the intratumoral injection provides an approach that does not require ex vivo Ag loading of DCs. Ags derived from dying tumor cells in chemotherapy-treated animals are taken up by DCs injected into the tumor. Danger signals at the site of dying tumor cells stimulate the DCs, resulting in their activation in situ and cross-priming of T cells against tumor Ags. This approach has been tested in a variety of murine solid tumor models (43–45) and in a murine lymphoma model (46). The present study extends this approach to a plasma cell tumor.

The s.c. injection of Id-KLH-loaded DCs, s.c. injection of tumor cell-loaded DCs, and intratumoral injection of naive DCs were similarly effective in mediating robust long-term regression of tumors producing intact Ig molecules. However, whereas tumors consisting of Id-negative cells or mixed populations of Id-positive and Id-negative cells could be cured by s.c. injection of tumor cell-loaded DCs or by intratumoral injection of naive DCs, they were not affected by s.c. injection of Id-KLH-loaded DCs. Analysis of Ig synthesis revealed that the Id-negative cells lost expression of the Ig H chain, but maintained production and secretion of the L chain. We demonstrated that tumor protection following immunization with the myeloma Ig was dependent on H chain expression by the tumor. This indicated that the anti-Id response was directed against epitopes residing in the Ig H chain. It was therefore not surprising that vaccination with Id-KLH-loaded DCs had no therapeutic effect on Id-negative variant tumor cells. Regression of these tumors following intratumor injection of DCs or s.c. injection of tumor cell-loaded DCs apparently resulted from a response to Ags other than Ig.

The present study has implications for DC vaccination in multiple myeloma patients. Targeting the Id in myeloma depends on efficient cell-mediated immune responses because, unlike anti-Id Abs, T cells would not be blocked by the large amounts of circulating paraprotein and would not depend on expression of the native protein on the tumor surface. Id-specific T cells respond to peptides corresponding to the CDRs of the monoclonal Ig. Although peptides derived from L chain CDRs may express T cell peptides, anti-Id T cell reactivity is mostly directed against peptides derived from H chain CDRs, especially from the V-J-D-J(3) (VH-D-JH) CDR3 region. The predominance of immunity against H chains has been observed in many Id-vaccination studies in multiple myeloma and lymphoma patients (47–51). This general predominance of Id-specific T cell responses directed against sequences of H chain CDRs, especially CDR3, is attributed to the much larger versatility of H chains, which is mainly determined by their D segment (49). Thus, in a recent clinical trial of DC vaccination in multiple myeloma patients, VDJ-derived peptides were as effective as the whole Id protein in stimulating T cell responses (18). Although most multiple myeloma cells are characterized by a monoclonal Ig, in up to 20% of myeloma cases only L chain is detected due to absence of H chain synthesis. These patients are commonly referred to as having L chain disease. Given that T cells respond primarily to H chain CDRs, attempts to treat L chain disease with myeloma protein-pulsed DCs may be futile. Indeed, our study demonstrated that vaccination with Id-loaded DCs had no therapeutic effect on tumors that secrete L chain only. However, s.c. injection of tumor cell-loaded DCs or intratumoral injection of naive DCs led to complete long-term regression of L chain-secreting tumors. These results suggest that vaccination with tumor cells loaded onto DCs may induce an effective antitumor response in patients with L chain disease. Treatment with tumor-loaded DCs may also be beneficial for patients with nonsecretory myeloma, and for myeloma patients whose Ig V(DJ) sequences do not contain peptide-binding motifs for MHC molecules, thus are not expected to develop Id-specific T cell responses.

The antitumor response induced by the tumor cell-DC vaccine did not depend on expression of Id by the tumor. It appears that one or more non-Id Ags served as targets for the induced response. These Ags have not yet been identified. However, several putative Ags in myeloma cells can serve as such targets. These include MUC-1 and cancer-testis Ags such as MAGE and NY-ESO-1 (52). Which of these Ags may be targeted by myeloma cell-DC vaccines remains to be determined.

Disclosures

The authors have no financial conflict of interest.

References


